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OF *PINUS TAEDA* L.**

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ABSCISIC ACID LEVELS IN EMBRYOS AND MEGAGAMETOPHYTES OF *PINUS TAEDA* L.

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Abstract. -- Absciscic acid (ABA) was quantified in developing zygotic embryos and megagametophytes of loblolly pine using indirect ELISA. On a per tissue basis, the amount of ABA present in the megagametophyte remained relatively constant at approximately 2.5 ng/megagametophyte. The ABA in embryos remained low during early to mid development, peaked to approximately 1.5 ng/embryo, before declining and peaking a second time prior to cone ripening. On a g dry weight basis, ABA in megagametophytes exhibited a steady drop from approximately 1750 ng in early development to 127 ng at cone ripening. ABA in embryos was high during early development (2250 ng/g dry weight) and displayed two significant peaks middle to late development before dropping to approximately 600 ng/g dry weight at cone ripening. Peak levels of ABA in embryos (on a tissue basis) appear to occur shortly before the increase in dry weight accumulation.

Key words: ELISA, loblolly pine, zygotic embryogenesis, somatic embryogenesis, seed maturation.

INTRODUCTION

Endogenous abscisic acid (ABA) has been implicated as a factor involved in regulating seed development and maturation. Putative roles of ABA in seeds during embryogenesis include inhibition of precocious germination, promotion of storage protein synthesis, suppression of reserve mobilization, promotion of desiccation tolerance, and induction of dormancy (Black 1991). In conifer somatic embryogenesis, exogenous ABA shows similar critical functions: inhibition of precocious germination and accumulation of storage nutrients, lipids, and proteins in embryos cultured on media containing specific levels of ABA in *Picea glauca* (Attree et al. 1992), *Picea glauca engelmanni* (Roberts 1991), *Picea abies* (Hakman et al. 1990), and *Pinus taeda* (Becwar and Feirer 1989). Enhancing lipid biosynthesis using ABA and polyethylene glycol improved desiccation tolerance in somatic embryos of *Picea glauca* (Attree et al. 1992).

Although ABA plays a critical role in the development of conifer zygotic and somatic embryos, very little is known about the biochemical and molecular events that occur during conifer zygotic embryogenesis. This incomplete knowledge makes the development of somatic embryogenesis difficult, particularly for loblolly pine, which has proven difficult to manipulate in culture. Usable embryos and seedlings have been produced, but successes remain sporadic and highly dependent on genotype (Gupta and Durzan 1987). Trial and error experimentation has been the hallmark of research on this technology, making it time-consuming and labor-intensive efforts.

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The objective of our research has been to use zygotic embryogenesis as a model to determine optimal ABA requirements for the somatic system. Concentration changes of ABA were measured in embryo, megagametophyte, and suspensor tissues during zygotic development. These trends will be used to adjust those used for somatic embryogenesis. Using the zygotic system as a model should make research to develop somatic embryogenesis more effective and efficient.

MATERIAL AND METHODS

Chemicals and Supplies. Buffers. PBS/Tween: 13.6 g KH_2PO_4 , 29.2 g NaCl per liter containing 0.05% Tween 20 (buffer adjusted to pH 7.4); blocking solution: 5% (w/v) bovine serum albumin (BSA) (Sigma, A-3425) in PBS/Tween; assay buffer: 0.1% (v/v) BSA in PBS/Tween. ABA-4'-BSA Conjugate. The conjugate was prepared according to Quarrie and Galfre (1985) and stored at -20°C in its concentrated form. The conjugate was diluted in 0.05 M NaHCO_3 (pH 9.6) to a concentration (1:100,000) determined by optimization trials. Monoclonal Antibody. The monoclonal antibody (mAb) raised against free *cis*-(+)-ABA (Mertens et al. 1983) was from Idetek, Inc., San Bruno, CA. The mAb was dissolved in 2 mL 0.1% BSA, aliquoted into 10 μL portions and stored at -80°C . The monoclonal was further diluted in assay buffer to a concentration (1:5000) as determined by optimization trials. Goat Anti-mouse Antibody-Biotin Conjugate. Goat anti-mouse antibody-biotin conjugate (Sigma, B-7264) was aliquoted into 10 μL portions and stored at -20°C . The conjugate was diluted 1:5000 in assay buffer prior to use. Streptavidin-Poly-HRP Conjugate. Streptavidin-Poly-HRP (Research Diagnostics, Inc., Flanders, NJ, No. RDI-pHRP20-SA) was aliquoted into 10 μL portions and stored at -20°C . The conjugate was diluted 1:5000 in assay buffer prior to use. Tetramethylbenzidine (TMB) Substrate. A 10 mg/mL solution of TMB (Sigma, T-2885) in DMSO was diluted 1:1000 in 100 mM $\text{Na}_2\text{H}_2\text{O}_2$ (pH 5.5). Hydrogen peroxide (3% solution) was added to a final concentration of 0.002%. Abscisic Acid (ABA) Standards. A stock solution of 50 mM (+)-*cis*-ABA was prepared by dissolving (\pm)-*cis,trans*-ABA (Sigma, A-1012) in absolute methanol. This solution was stable for at least three months when stored at -20°C and darkness. Standards ranging from 15.8 ng to 0.25 pg/100 μL (+)-ABA were made in assay buffer by serial dilution. The small amount of methanol in the standards did not affect the performance of the antibody (data not presented). Microplates. Immulon-2, flat-bottom, 96-well microtitration plates (Dynatech Laboratories, Chantilly, VA) were utilized for optimal binding of the ABA-4'-BSA conjugate (Walker-Simmons 1987). Microplate Reader and Washer. Absorbencies were read using a Bio-Tek Kinetic Reader Model EL312E at 630 nm and 450 nm wavelengths. Micro-plates were aspirated and washed using a Bio-Tek manual washer Model EL40112.

Plant Material. Loblolly pine cones from four seed orchards (generously provided by Westvaco Corp., Weyerhaeuser Co., and Union Camp Corp.) were shipped overnight, on ice packs, on a weekly basis from June to October 1992 (fertilization to cone ripening). Clones were designated "WV" for Westvaco, "WA" and "WB" for Weyerhaeuser, and "UC" for Union Camp. Seeds were immediately extracted from the cones upon delivery, and embryos, suspensors, and megagametophytes were separated under a stereoscopic microscope. The embryos were staged for morphological development and briefly rinsed in distilled water to remove extraneous hormones that may have leaked from the megagametophyte during the dissection. The three tissues were frozen separately in liquid nitrogen, and the collected material was then lyophilized and stored at -80°C until analyzed. Tissues were thus stored based on clone, tissue type, and stage of development.

Staging of Developing Embryos. Staging of the tissues was based on morphological changes of the developing embryos using a method developed at IPST (Webb 1991). Stage 1: proembryos, from free nucleate to 12 cells; Stage 2: embryo proper, microscopic but distinct/translucent, still found at micropylar end; Stage 3: embryo proper becomes white/opaque and is found at chalazal end; Stage 4: larger than Stage 3, opaque embryo proper enlarged longitudinally, dome shaped apex; Stage 5: similar to Stage 4, except apical meristem is visible; Stage 6: similar to Stage 5, except cotyledon primordia are barely visible below apical meristem; Stage 7: similar to Stage 6, except cotyledons are elongated, but do not overtop apical meristem; Stage 8/8B: similar to Stage 7, except cotyledons overtop apical meristem, apical meristem still visible; Stage 9: cotyledons curved and joined at their tips; apical meristem is not visible. Stage 9 tissue was subdivided by percent moisture content of tissue since growth continued without further obvious morphological change (9A-9I).

ABA Analysis. ABA Extraction. Isolated tissues were ground using a cold glass rod, weighed, and extracted in 80% methanol (v/v) containing 25 mg/L butylated hydroxytoluene (BHT) adjusted to pH 7. Approximately 10^5 DPM of [3 H]-ABA (Amersham, Arlington Heights, IL, TRK.644) was added as an internal standard. Tissues were extracted overnight with stirring at 4°C in the dark.

ABA Purification. The homogenate was centrifuged at 2000 g for 15 minutes and the supernatant removed. The pellet was resuspended in 1 mL of extracting solvent and recentrifuged at 2000 g for 10 minutes. The supernatants were pooled, passed through a 0.45 μ m nylon filter, and reduced to near dryness using rotoevaporation in vacuo at 35°C and dim lighting. The remaining aqueous phase was diluted to 1000 μ L in assay buffer for analysis by indirect ELISA. This procedure gave an optimum extraction of ABA of 92% (95% CI 2.5%) recovery.

ABA Quantification. This procedure is modified from Walker-Simmons (1987) by addition of a biotin-streptavidin-multiple horseradish peroxidase (HRP) system for amplification. **Coating of microtiter plate.** Diluted ABA-4'-BSA conjugate (200 μ l) were added to each well of the microtiter plate except those in the outside rows as they have been shown to produce inconsistent results (Ross et al. 1987). The plates were sealed with Parafilm and incubated overnight at 4°C in the dark. **Incubation of standards or samples with ABA antibody.** ABA standards and samples were diluted 1:1 with the diluted monoclonal antibody and incubated overnight at 4°C in the dark. Triplicate standard curves were included on every plate. Samples were diluted in assay buffer to obtain absorbencies in the center of the calibration curve. **Blocking of the wells.** Plates were aspirated and washed four times with PBS/Tween. Approximately 300 μ L of blocking solution were added to each well, and left to incubate for 45 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS/Tween. **Addition of standard or samples plus monoclonal to plate.** Samples or standards plus monoclonal antibody (200 μ l) were added to the plate, which was then incubated for 90 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS/Tween. **Addition of goat anti-mouse antibody-biotin conjugate.** Diluted antibody-biotin conjugate (200 μ l) were added to each well, and the plate was incubated for 90 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS/Tween. **Addition of Streptavidin-Poly-HRP conjugate.** Diluted streptavidin-Poly-HRP conjugate (200 μ l) was added to each well, and the plate was incubated for 90 minutes at 37°C in the dark. The wells were aspirated, washed four times with PBS/Tween, and once with 300 μ L/well of 100 mM NaC₂H₃O₂ to remove potassium salt. **TMB reaction.** TMB solution (200 μ l) was added to each well, and the blue color was allowed to develop at room temperature in the dark until a B₀ absorbance of approximately 0.40 at 630 nm was obtained. Forty microliters of a 1.5 N H₂SO₄ solution were added to each well to stop the reaction, and the absorbance was read at 450 nm. A logit transformation was performed on the absorbencies of the standards to linearize the calibration curve. The

quantity of (+)-ABA in the samples was calculated by performing the logit transformation and comparing the results to the calibration curve of (+)-ABA for each plate.

GC/MS-SIM Validation of the indirect ELISA. Samples extracted for validation of the indirect ELISA were purified as stated above and taken to complete dryness using rotoevaporation in vacuo at 37°C and dim lighting. Samples were methylated with ethereal diazomethane, and ABA levels were quantified by GC/MS-SIM using $^2\text{H}_6$ -ABA as an internal standard. The analysis was performed on a Hewlett-Packard 5890 GC with capillary direct interface to a Hewlett-Packard 5971A mass-selective detector. The GC program was 60°C for 2 min., 25°C/min. to 165°C, then 5°C/min. to 275°C. The helium gas flow was 0.5 mL/min. The capillary column was a DB1 (J&W Scientific, Ltd.) 15 m x 0.25 mm x 0.25 μm film. The injection and interface temperatures were 300°C. Data were collected using the SIM program, monitoring four ions: m/e 190 (endogenous ABA), m/e 194 ($^2\text{H}_6$ -ABA), and m/e 162 and 166 to monitor impurities. Retention time for $^2\text{H}_6$ -ABA was 12.34 min. and for ABA was 12.37 min. A calibration curve for $^2\text{H}_6$ -ABA was prepared by monitoring the 190/194 ratios for 308 ng $^2\text{H}_6$ -ABA in standard samples of ABA of 50, 100, 200, 400, 600, 800, and 1000 ng. The amount of ABA in the samples was estimated by entering the 190/194 ratio peak areas into the regression equation of the calibration curve.

Statistical Analysis. Statistical significance of the multiple pairwise comparisons for ABA in the tissues was based on Bonferroni-Welch which assumes normal distributions but unequal variances between samples. Analysis was carried out at 95% confidence intervals ($\alpha=0.05$).

RESULTS AND DISCUSSION

ABA Levels in Developing Seeds

ABA content is usually expressed on a per tissue or on a concentration basis (i.e., dry or fresh weight). Both techniques have advantages and limitations. In this study, concentration on a dry weight basis was chosen due to the necessity of freezing and storing the tissues as quickly as possible. "Per tissue" basis results were determined by dividing total dry weight by the number of embryos or megagametophytes collected (i.e., "average" embryo or megagametophyte basis).

Indirect ELISA was selected as the method of analysis because of its high specificity and sensitivity toward ABA (1 pg/100 μL), and due to the small amounts of embryo tissue (< 0.3 mg dry weight) that were available in the early stages of development (Stages 3-7). ELISA validation by GC/MS-SIM using late stage tissue demonstrated good agreement between the two methods, and showed no crossreactants or interferents to the ELISA (data not presented).

Changes in ABA levels found in developing embryos and megagametophytes are illustrated for "UC" in Figures 1 and 2 on a per tissue and dry weight basis, respectively. ABA was detected in all tissues tested. In many herbaceous species, ABA has been found to be extremely low or undetectable during early development, increasing to a maximum level at one-third to one-half maturation with subsequent decreases back to low levels at maturity (Black 1991). This trend was not found on a per tissue basis in loblolly pine. There were no significant changes in ABA found in megagametophytes during development, except for tissue at Stages 9A and 9B which were significantly higher. In embryos, there was a significant increase in ABA from Stages 8 to 9A, followed by a decline and a second increase at 9F. The first increase was simultaneous with the apparent increase in the megagametophyte, inferring ABA in embryos was imported from the megagametophyte. In other species, it is not known whether the measured ABA was synthesized by

seed tissues or imported from the mother plant (Brenner 1986). ABA in the megagametophyte was significantly higher than in the corresponding embryo tissue, a trend that appears to be species-dependent. In *Zea mays* L., ABA in the embryo was found to be higher than in the endosperm (Jones and Brenner 1987), while the opposite was found in (*Prunus persica* L.) (Piaggese et al. 1991).

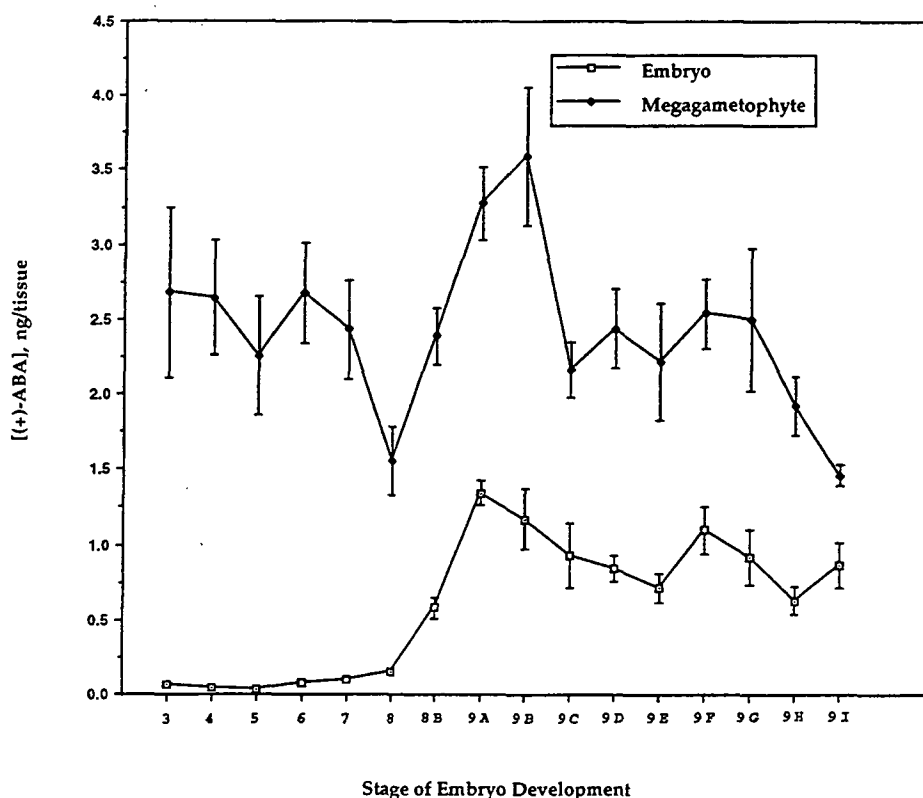


Figure 1: ABA levels in developing embryos and megagametophyte from the "UC" from stage 3 to cone ripening (June-October 1992), on a per "tissue" basis. Stage 9 tissue was divided into additional substages according to percent moisture content of embryo/megagametophyte, respectively: A: 62/42%, B: 57/38%, C: 44/31%, D: 35/30%, E: 34/28%, F: 30/25%, G: 32/24%, H: 31/25%, I: 30/25%. Error bars represent 95% confidence limits.

The generalization reported by Black (1991) does not apply for ABA per dry weight as well (Figure 2). Overall, the highest levels of ABA in embryos were found at early development with a steady decline to cone ripening. There appears to be at least two significant peaks (Stages 7 and 9F), possibly four (Stages 4 and 9A) for ABA in embryos of loblolly pine. Multiple peaks for ABA are not uncommon, and it has been reported that endogenous ABA levels can exhibit two peaks in embryos of developing *Phaseolus coccineus* (Hsu 1979) and *P. vulgaris* L. (Perata et al. 1990) and three to four peaks in *Brassica napus* L. (Finkelstein et al. 1985). However, these peaks do not necessarily occur at the same point during seed development.

In the work to date with "UC", the ABA peaks do not appear to correspond to any major morphogenic changes in the embryos, e.g., development of cotyledons. The ABA determined in embryos is significantly higher than that in the megagametophyte tissue. Again, this trend appears to be species-dependent. Although similar trends have been demonstrated with *Hordeum vulgare* L. (Quarrie et al 1988) and *Zea mays* L. (Jones and Brenner 1987), the opposite was found in *Medicago sativa* L. (Xu et al. 1990). It would appear that ABA in

loblolly pine megagametophytes does not change (Figure 1), and the decline in ABA depicted in Figure 2 is caused by an increase in dry weight during development rather than by a drop in ABA.

ABA Levels and Dry Weight Accumulation in Developing Embryos

Changes in embryo ABA relative to dry weight displayed in Figure 3. A rapid increase in ABA occurred just prior to that in dry weight. This trend has been observed in many species, including *Triticum*, *Bassica*, and *Glycine* (Black 1991) and *Zea* (Jones and Brenner 1987). Although a cause-and-effect relationship is suggested, the increase in ABA levels cannot be said to cause the increase in loblolly pine embryo dry mass without further evidence.

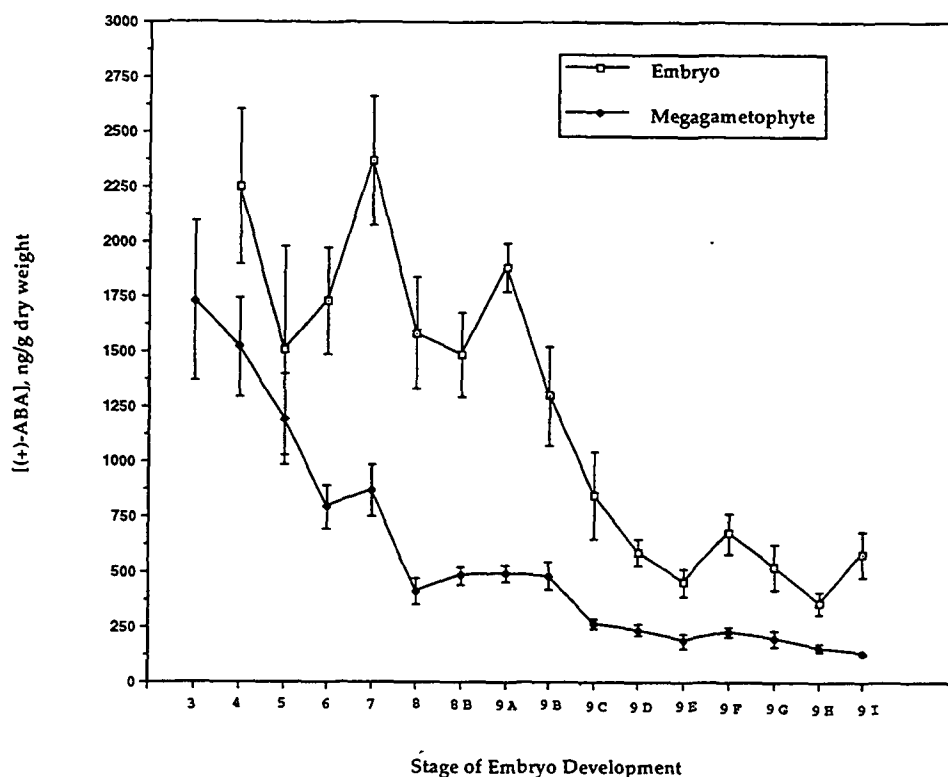


Figure 2. ABA levels in developing embryos and megagametophyte from the "UC" from Stage 3 to cone ripening (June-October 1992), on a per g dry weight basis. Stage 9 tissue was divided into additional substages according to percent moisture content. See Figure 1 for description of substages. Error bars represent 95% confidence limits.

SUMMARY AND CONCLUSION

Results for the single clone analyzed to date suggest that ABA in megagametophytes (on a tissue basis) remains constant during seed development and maturation. ABA in embryos remained low during early to middle stages of development, increased to its highest level at Stage 9A, then steadily declined and peaked a second time at 9F. Changes in embryo ABA levels did not appear related to any major morphogenic event, but the major increase observed midway through development preceded exponential growth and dry weight accumulation.

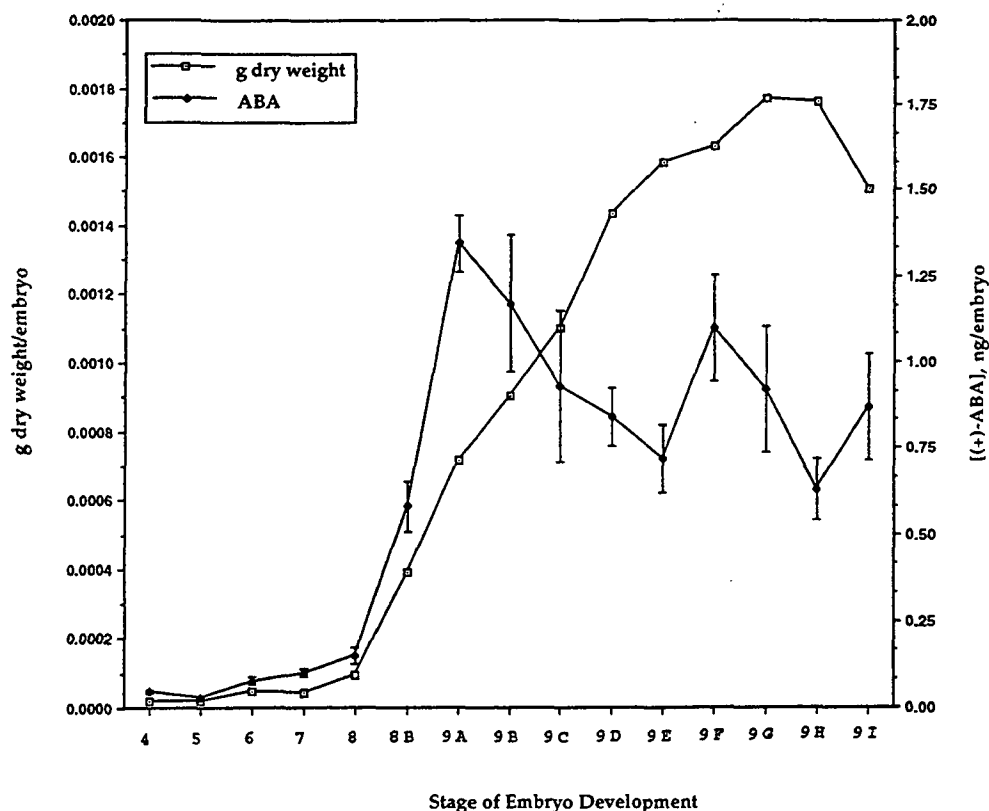


Figure 3. ABA level versus dry weight accumulation in developing embryos from the "UC" from stage 3 to cone ripening (June-October 1992). Stage 9 tissue was divided into additional substages according to percent moisture content. See Figure 1 for description of substages. Error bars represent 95% confidence limits.

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